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Differential Gene Expression Profiling of Mouse Uterine Luminal Epithelium During Periimplantation

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Abstract

Uterine luminal epithelium (LE) is critical for establishing uterine receptivity. Microarray analysis of gestation day 3.5 (D3.5, preimplantation) and D4.5 (postimplantation) LE from natural pregnant mice identified 382 upregulated and 245 downregulated genes in the D4.5 LE. Gene Ontology annotation grouped 186 upregulated and 103 downregulated genes into 22 and 15 enriched subcategories, respectively, in regulating DNA-dependent transcription, metabolism, cell morphology, ion transport, immune response, apoptosis, signal transduction, and so on. Signaling pathway analysis revealed 99 genes in 21 significantly changed signaling pathways, with 14 of these pathways involved in metabolism. In situ hybridization confirmed the temporal expression of 12 previously uncharacterized genes, including *Atp6v0a4*, *Atp6v0d2*, *F3*, *Ggh*, *Tmprss11d*, *Tmprss13*, *Anpep, Fxyd4*, *Naip5*, *Npl*, *Nudt19*, and *Tpm1* in the periimplantation uterus. This study provides a comprehensive picture of the differentially expressed genes in the periimplantation LE to help understand the molecular mechanism of LE transformation upon establishment of uterine receptivity.

Keywords

microarray analysis, uterine luminal epithelium, uterine receptivity, embryo implantation

Introduction

Uterine receptivity refers to a transient state in which the maternal endometrium is receptive for an embryo to implant.[1-4] Ovarian hormones progesterone (P4) and estrogen (E2) are the master controls for the establishment of uterine receptivity in mammals.[5-8] The ovarian hormone-regulated uterine receptivity is restricted and transient. Since such restriction is abolished if the uterine luminal epithelium (LE) is broken or absent, LE is considered essential for the receptive sensitivity of the uterus.[9,10] In addition, LE plays a transmitter role for decidualization,[11] a critical response in the stromal compartment at the implantation site upon implantation initiation. The importance of LE in the establishment of uterine receptivity is also manifested by its physical interaction with the implanting embryos during the initial stages of embryo implantation, embryo apposition to the LE, embryo adhesion to the LE, and embryo penetration through the LE.[1,12-14]

It has been observed that the LE ultrastructure, such as LE cell surface components, lateral adherent junctions and gap junction channels, and subepithelial extracellular matrix (ECM), changes during the establishment of uterine receptivity.[15-20] The morphological transformations are likely associated with the differential gene expression in the LE, for example, upregulation of gap junction protein β-2 (GJB2/Cx26) in the LE likely contributes to the altered gap junction channels in the LE.[21] Differential gene expression of many genes in the periimplantation LE has been reported, such as cytochrome P450 26A1 (*Cyp26a1*),[22] *Gjb2,*[21] histidine decarboxylase (*Hdc*),[23] leukemia inhibitory factor receptor (*Lifr*),[24] lysophosphatidic acid receptor 3 (*Lpar3*),[25] msh homeobox 1 (*Msx1*),[26] myeloid differentiation primary response gene 88 (*MyD88*),[27] ethanolamine kinase 1 (*Etnk1*),[28] progesterone receptor (*Pgr*),[29-31] phospholipase A2, group IVA (*Pla2g4a*),[32] proline-rich acidic protein 1 (*Prap1*),[33] wingless-related MMTV integration site 7B (*Wnt7b*),[34] and so on. However, the knowledge of global molecular transformation in the LE during periimplantation is still incomplete.

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Microarray analysis has been widely used to determine the gene expression profiling associated with implantation in the mouse uterus[30,35,36] and in the LE.[27,28,37] The LE cells represent only 5% to 10% of total uterine cells.[27,37] It is possible that some significantly changed gene expression in the LE may be obscured in the whole uterus microarray.[30,35] Several studies have focused on LE gene expression.[27,28,37] However, the data from these studies could not reflect the gene expression profiles in the LE from natural pregnant periimplantation uteri. One study used LE from ovarian hormone P4- and E2-treated nonpregnant ovariectomized mice to replicate the window of uterine receptivity[27]; one study compared the gene expression profiles between LE and glandular epithelium (GE) from P4- and E2-treated ovariectomized early pregnant mice (delayed implantation model)[28]; and the third study compared the gene expression profiles in the postimplantation LE at implantation site and interimplantation site in delayed implantation mouse model.[37] In this study, LE cells were isolated from preimplantation D3.5 and postimplantation D4.5 natural pregnant mouse uteri. The gene expression profiles in the periimplantation LE from natural pregnancy will provide us with a comprehensive picture about the molecular transformation of LE during the establishment of uterine receptivity.

Materials and Methods

Animals

C57BL6/129svj-mixed background wild-type (WT) mice were generated from a colony at the University of Georgia.[25] Mice were housed in polypropylene cages with free access to regular food and water from water sip tubes in a reverse osmosis system. The animal facility is on a 12-hour light/dark cycle (6:00 AM to 6:00 PM) at 23 $\pm$ 1°C with 30% to 50% relative humidity. All methods used in this study were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.

Mating, Uterine Tissue Collection, Isolation of Uterine LE, Total RNA Isolation

Young virgin females (2-4 months old) were mated naturally with WT stud males and checked for a vaginal plug the next morning. The day a vaginal plug identified was designated as gestation day 0.5 (D0.5, mating night as D0). Uterine tissues were collected from euthanized females between 11:00 and 12:00 hours on D3.5 and D4.5, respectively. About one-third of a uterine horn from each euthanized female was frozen on dry ice. The remaining uterine tissue was processed for LE isolation as previously described using 0.5% dispase enzyme and gentle scraping.[38] The isolated LE sheets from D3.5 and D4.5 uteri were subjected to total RNA isolation using TRIzol (Invitrogen, Carlsbad, California). The pregnancy status was determined by the presence of blastocysts in the D3.5 uterus or implantation sites in the D4.5 uterus. At least 3 pregnant mice were included in each group.

Microarray Analysis

Microarray analysis was performed at the Emory Biomarker Service Center, Emory University using Affymetrix_Mouse Gene 1.0 ST Chip covering 28,853 genes. Three replicates from different mice were included in each group. Microarray data (GEO number: GSE44451) were analyzed using GeneSpring 12.1 GX (Agilent Technologies, Santa Clara, California).[39] The negative and missing values were threshold to 1 before the log transformation. Percentile shift normalization was performed to overcome the difference among different arrays, and entries with the lowest 20 percentile of the intensity values were removed. The criteria for determining differential gene expression included a fold change of $\geq$2, P value of $< .05$, and an absolute mean difference in the intensity values of >200 between the 2 groups. Gene Ontology annotation and signaling pathway analysis were performed using DAVID Analysis and GeneSpring 12.1 GX, respectively.[40]

Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was used to validate selected genes from the microarray analysis. Total RNA from D3.5 and D4.5 whole-uterine horns was isolated using TRIzol. Complementary DNA (cDNA) was reverse transcribed from 1 μg of total RNA using Superscript III reverse transcriptase with random primers (Invitrogen). Real-time PCR was performed in 384-well plates using Sybr-Green I intercalating dye on ABI 7900 (Applied Biosystems, Carlsbad, California). Primer sequences are listed in Supplemental Table 1 (Integrated DNA Technology, San Diego, California).

In Situ Hybridization

In situ hybridization was performed as described previously.[31,33,38] Antisense and sense probes for Atpase, H+ transporting, lysosomal V0 subunit A4 (*Atp6v0a4*), Atpase, H+ transporting, lysosomal V0 subunit D2 (*Atp6v0d2*), coagulation factor III (*F3*), γ-glutamyl hydrolase (*Ggh*), transmembrane protease, serine 11d (*Tmprss11d*), transmembrane protease, serine 13 (*Tmprss13*), alanyl aminopeptidase (*Anpep*), FXYD domain-containing ion transport regulator 4 (*Fxyd4*), nod-like receptor family, apoptosis inhibitory protein 5 (*Naip5*), N-acetylneuraminate pyruvate lyase (*Npl*), nucleoside diphosphate linked moiety X-type motif 19 (*Nudt19*), tropomyosin 1 (*Tpm1*), tropomyosin 2 (*Tpm2*), tropomyosin 3 (*Tpm3*), and tropomyosin 4 (*Tpm4*) were synthesized from cDNA fragments amplified with their respective gene-specific primer pairs (Supplemental Table 1).

Statistical Analyses

Two-tail unequal variance Student t test was used to compare the messenger RNA (mRNA) expression levels. The significant level was set at $P < .05$.

Table 1. Signaling Pathways Changed in the Periimplantation Mouse Uterine Luminal Epithelium.

		Significantly Changed Genes	
Signaling Pathways	*P* Value	Upregulated	Downregulated
Fattyacid biosynthesis	6.11E−06	*Acly, Acsl4, Echs1*	*Acaca, Acsl2, Fasn, Pcx*
Glycolysis and gluconeogenesis	2.14E−04	*Eno1, Pkm2, Slc2a1*	*Pcx, Slc2a3*
Purine metabolism	2.58E−04	*Entd5, Gda, Gmpr, Nt5e, Pde4d, Pkm2, Pnp, Pnp2, Polr1a, Polr1b, Prps2*	*Adcy9, Nt5c2*
Metapathway biotransformation	2.59E−04	*Chst12, Comt1, Cyp26a1, Fmo2, Mgst1*	*Gpx1, Cyp2s1, Hs3st1*
Triacylglyceride synthesis	9.64E−04	*Agpat5, Gpam*	*Ppap2b*
Insulin signaling	1.11E−03	*Eif4ebp1, Igf1r, Mapk6, Prkaa1, Prkaa2, Sgk, Xbp1*	*Pik3r3, Slc2a1,*
One carbon metabolism and related pathways	1.55E−03	*Ahcyl1*	*Chdh, Chpt1, Etnk1, Gpx1, Pcyt1b, Tyms*
Amino acid metabolism	1.61E−03	*Acly, Glud, Hal, Srm, Pkm2*	*Adh5, Hdc, Pcx*
α-6-β-4 integrin signaling pathway	2.08E−03	*Dst, Eif4ebp1, Lama3, Met, Mmp7, Sfn*	*Pik3r3*
Prostaglandin synthesis and regulation	3.88E−03	*Anxa3, Ptger2, S100a10*	*Pla2g4a, Ptgdr*
TCA cycle	3.88E−03	*Idh2, Idh3a*	*Pcx*
Complement and coagulation cascades	4.88E−03	*C3, F3, Cfh, Kng1*	*C2*
Kennedy pathway	1.12E−02		*Chpt1, Etnk1, Pcyt1b*
Wnt signaling pathway and pluripotency	1.68E−02	*Ccnd1, Mmp7, Myc, Ppp2r1b, Tcf4, Wnt7b*	*Fzd6*
Adipogenesis	1.69E−02	*Cebpa, Cyp26a1, Klf7, Lifr, Mif*	*Gata2, Zmpste24*
miRNA regulation of DNA damage response	2.43E−02	*Ccnd1, Sfn, Trp53*	*Ddb2, Sesn1*
Nucleotide metabolism	2.62E−02	*Prps2, Srm*	*Mthfd2*
Fatty acid β oxidation	2.86E−02	*Acsl4, Cpt1a, Echs1*	*Acss2*
Urea cycle and metabolism of amino groups	3.00E−02	*Glud1, Srm*	*Ckb*
Regulation of actin cytoskeleton	3.66E−02	*Enah, Fgf9, Itga1, Mapk6, Myh10, Pip5k1b*	*Pik3r3, Rdx*
EGFR1 signaling pathway	4.19E−02	*Cblb, Cebpa, Eps8, Myc*	*Pik3r3, Plscr1*

Abbreviations: EGFR1, epidermal growth factor receptor 1; miRNA, micro RNA.

Results

Categorization of Differentially Expressed Genes in the Periimplantation LE

Microarray analysis indicated 382 significantly upregulated genes and 245 significantly downregulated genes in the postimplantation D4.5 LE compared with that in the preimplantation D3.5 LE. The most upregulated 10 genes in the D4.5 LE were *Atp6v0d2* (34.70x), *Ggh*, *Prap1*, tocopherol transfer protein (α; *Ttpa*), tolloid-like protein 1 (*Tll1*), γ-aminobutyric acid A receptor, pi (*Gabrp*), matrix metallopeptidase 7 (*Mmp7*), glutathione peroxidase 3 (*Gpx3*), interferon induced transmembrane protein 6 (*Ifitm6*), and olfactomedin 1 (*Olfm1*). The most downregulated 10 genes in the D4.5 LE were *Npl* (35.42x), calbindin 1 (*Calb1*), G protein-coupled receptor 128 (*Gpr128*), solute carrier family 2, member 3 (*Slc23a*), UDP-glcnac: betagal β-1,3-N-acetylglucosaminyltransferase 5 (*B3gnt5*), interleukin 17 receptor B (*Il17rb*), *Fxyd4*, carbonyl reductase 2 (*Cbr2*), transmembrane protein 158 (*Tmem158*), and acyl-coA thioesterase 7 (*Acot7*). A complete list of all significantly changed genes with functional subcategory, Gene Ontology term, gene description, accession number, and fold change is shown in Supplemental Table 2 for upregulated genes in the D4.5 LE and in Supplemental Table 3 for downregulated genes in the D4.5 LE, respectively.

Gene Ontology annotation was conducted to categorize the differentially expressed genes based on the biological processes (Figure 1). Among the 382 significantly upregulated genes in the D4.5 LE, 187 (48.95%) genes were classified into 22 subcategories, and the remaining 195 (51.05%) genes were ungrouped. Seven largest categories with over 10 significantly upregulated genes were DNA-dependent transcription (7.85%, 30 genes), proteolysis (5.50%), transmembrane transport (5.24%), homeostatic process (3.93%), oxidation–reduction process (3.40%), lipid biosynthetic process (3.14%), and regulation of cell adhesion (3.14%; Figure 1A; Supplemental Table 2). Among the 245 downregulated genes in the D4.5 LE, 103 (42.04%) genes were grouped into 15 subcategories, including DNA-dependent transcription (7.35%, 17 genes), ion transport (4.49%), phosphorylation (4.49%), chromosome organization (3.67%), and so on, and the remaining 142 genes (57.96%) were ungrouped (Figure 1B; Supplemental Table 3). Interestingly, DNA-dependent transcription was the most enriched subcategory for both the upregulated and the downregulated genes in the D4.5 LE, in which transcription factors *Arnt2* (Aryl hydrocarbon receptor nuclear translocator 2) and *Myc* (myelocytomatosis oncogene) were the most upregulated genes and *Pgr* (progesterone receptor) was the most downregulated gene (Figure 1; Supplemental Tables 2 and 3). The following 8 subcategories, proteolysis, transmembrane transport, homeostatic process, oxidation–reduction process,

Table 2. Comparison of the Differentially Expressed Genes in the 3 References That Overlap With the Differentially Expressed Genes (Total 627) in This Study (D3.5 LE vs D4.5 LE).

Reference	Reference[27]	Reference[28]	Reference[7]
Mouse model	Ovariectomized mice treated with E2 or E2 + P4	Delayed implantation	Delayed implantation
Comparison	E2-treated LE vs E2 + P4-treated LE	LE vs GE	IS LE vs inter-IS LE
No. of differentially expressed genes	222 (up in E2+P4-treated LE)	153 (up in LE) 118 (down in LE)	136 (up in IS LE) 223 (down in IS LE)
No. of overlapped genes with this study	19	28	20
Detail description	Up in E2 + P4-treated LE and up in D4.5 LE (4 genes): *H6pd*, *Igfbp3*, *Olfm1*, and *Mmp7* Up in E2 + P4-treated LE and down in D4.5 LE (15 genes): *Calb1*, *Fxyd4*, *Marcks*, *Jam2*, *Lrpap1*, *Lrp2*, *Nde1*, *Ovgp1*, *Rdx*, *Tmod2*, *Cln5*, *Pfkfb3*, *Serpina1e*, *Hdc*, and *Prune*	Up in LE and up in D4.5 LE (8 genes): *Bcap29*, *Atp11a*, *Nt5e*, *Tacstd2*, *Atp6v0a4*, *Blnk*, *Tgfbi*, and *Efhd1* Up in LE & down in D4.5 LE (12 genes): *Calb1*, *Fxyd4*, *Sorl1*, *Jam2*, *Nudt19*, *Car2*, *Chdh*, *Rdx*, *Cln5*, *Etnk1*, *Fasn*, and *Hdc* Down in LE and down in D4.5 LE (2 genes): *Slc2a3* and *Ces3* Down in LE and up in D4.5 LE (6 genes): *Lsyna1*, *Sult1d1*, *Ide*, *Enah*, *Ern1*, and *Manba*	Up in IS LE and up in D4.5 LE (8 genes): *Mgst1*, *Fads3*, *Gmpr*, *Hal*, *Pla2g7*, *Cfh*, *Wnt7b*, and *Ggh* Down in IS LE and down in D4.5 LE (11 genes): *Npl*, *Ank*, *Stx18*, *Lrpap1*, *Ckmt1*, *Ly75*, *Atp2b2*, *Myd88*, *Tmod2*, *Pmp22*, and *Plscr1* Down in IS LE and Up in D4.5 LE (1 gene): *Irf1*

Abbreviations: D3.5, gestation day 3.5, preimplantation; D4.5, gestation day 4.5, postimplantation; E2, 17β-estradiol; P4, progesterone; IS, implantation site; inter-IS, interimplantation site; LE, luminal epithelium; GE, glandular epithelium.

regulation of cell adhesion, establishment of protein localization, ion transport, and glycoprotein biosynthetic process, were shown in both the upregulated and the downregulated gene groups (Figure 1; Supplemental Tables 2 and 3).

Signaling Pathway Analysis of the Differentially Expressed Genes in the Periimplantation LE

In Gene Ontology annotation, each gene was grouped into a single subcategory (Figure 1; Supplemental Tables 2 and 3). However, in signaling pathway analysis, one gene could be clustered into multiple signaling pathways, for example, *Cyp26a1* was shown in both metapathway biotransformation and adipogenesis pathways (Table 1). Among the 627 differentially expressed genes in the periimplantation LE, 100 genes were classified into 25 significantly changed signaling pathways. Of the top 10 most upregulated genes in the D4.5 LE, 1 gene, *Gpx3*, was assigned into 6 of the 25 significantly changed signaling pathways. However, *Gpx3* was reported to be detected in the stromal compartment but not in LE of D4.5 uterus,[41] and we confirmed this expression pattern by in situ hybridization (data not shown). The *Gpx3* was thus removed from signaling pathway analysis. Without *Gpx3*, 99 differentially expressed genes were classified into 21 significantly altered signaling pathways in the periimplantation LE (Table 1). Fourteen (66.7%) of these signaling pathways were involved in metabolism that included the top 5 most significantly altered pathways, fatty acid biosynthesis ($P = 6.11E{-}06$), glycolysis and gluconeogenesis ($P = 2.14E{-}04$), purine metabolism ($P = 2.58E{-}04$), metapathway biotransformation ($P = 2.59\ E{-}04$), and triacylglyceride synthesis (9.64E−04; Table 1). Other metabolic pathways included 1 carbon metabolism and related pathways, amino acid metabolism, prostaglandin synthesis and regulation, TCA cycle, Kennedy pathway, adipogenesis, nucleotide metabolism, fatty acid β oxidation, and urea cycle and metabolism of amino groups (Table 1). The remaining 7 pathways were involved in insulin signaling, α-6-β-4 integrin signaling pathway, complement and coagulation cascades, Wnt signaling pathway and pluripotency, micro RNA (miRNA) regulation of DNA damage response, regulation of actin cytoskeleton, and epidermal growth factor receptor 1 signaling pathway (Table 1).

Gene Expression Confirmation by Real-time PCR and In Situ Hybridization

To validate microarray data, the mRNA levels of 7 upregulated and 7 downregulated genes were examined by real-time PCR and in situ hybridization. Among these 14 genes, *Prap1* and *Hdc* served as positive controls.[23,31,33] *Atp6v0a4* and the most upregulated *Atp6v0d2* in the D4.5 LE encode subunits for the vacuolar-type H^+-ATPase (V-ATPase), which is involved in transmembrane proton translocation.[42-44] *F3* encodes coagulation factor III, a cell surface glycoprotein involved in initiating coagulation pathway and inflammatory signaling.[45] *Ggh* encodes a lysosomal enzyme important for the cellular homeostasis of folate.[46] *Tmprss11d* and *Tmprss13* encode proteases.[47-49] *Anpep* is also called *CD13*, encoding a membrane protein that plays a role in digesting peptides and may also play roles in regulating keratinocyte-mediated ECM remodeling and fibroblast contractile activity[50] as well as immune responses.[51] *Fxyd4*

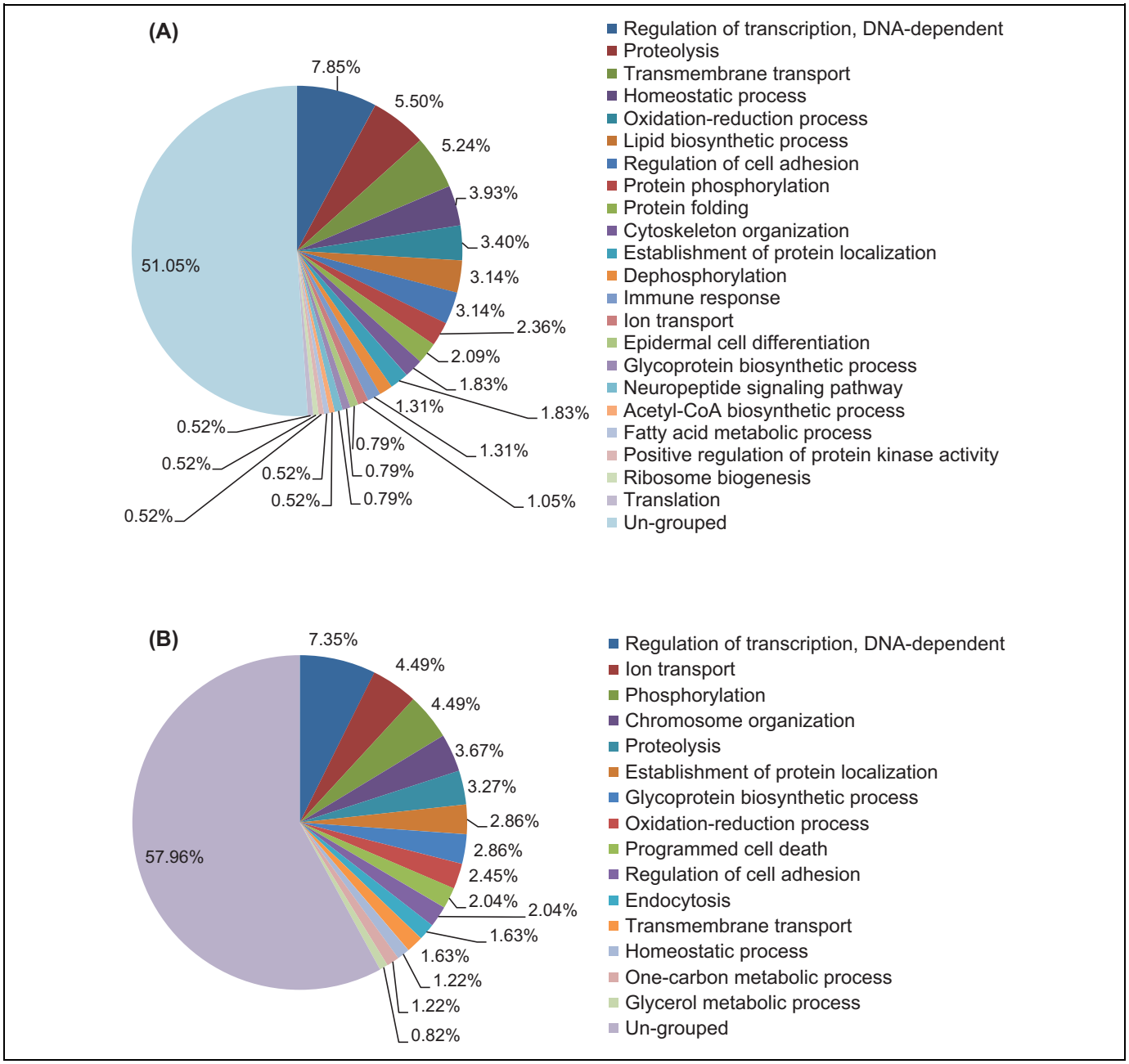

Figure 1. Categorization of genes whose transcript abundance is significantly changed in uterine LE upon embryo implantation via Gene Ontology Annotation. A, Pie chart of categorization (percentages) of genes significantly upregulated in the gestation day 4.5 (D4.5) LE. B, Pie chart of categorization (percentages) of genes significantly downregulated in the D4.5 LE. Only the genes with a minimal fold change of 2, $P < .05$, and an absolute value of mean difference greater than 200 between the 2 groups were included. N = 3 for both the groups.

encodes a regulator of the Na, K-ATPase.[52] *Naip5* is also called *Birc1e*, encoding an apoptosis inhibitory protein that can be upregulated by a synthetic estrogen diethylstilbestrol in the postnatal day 5 mouse LE.[53] The most downregulated gene, *Npl*, regulates cellular sialic acid concentration.[54,55] The *Nudt19*, also called *RP2*, hydrolyzes CoA esters[56] and possesses mRNA decapping activity.[57] The *Tpm1* is 1 of the 4 genes encoding tropomyosin, an actin-binding protein involved in smooth muscle contraction and mediating actin cytoskeleton functions in nonmuscle cells.[58,59]

Relative readings of these genes in the microarray analysis are shown in Figure 2A. The differential expression of these genes was confirmed by real-time PCR in the LE (Figure 2B) and in the whole uterus, in which only *Tmprss13* did not show significant difference (Figure 2C) but with similar trend of change as microarray data (Figure 2A) and LE real-time PCR (Figure 2B).

Consistent with microarray data and real-time PCR results (Figure 2; Supplemental Table 2), *Atp6v0a4*, *Atp6v0d2*, *F3*, *Ggh*, *Tmprss11d*, and *Tmprss13* were upregulated in the

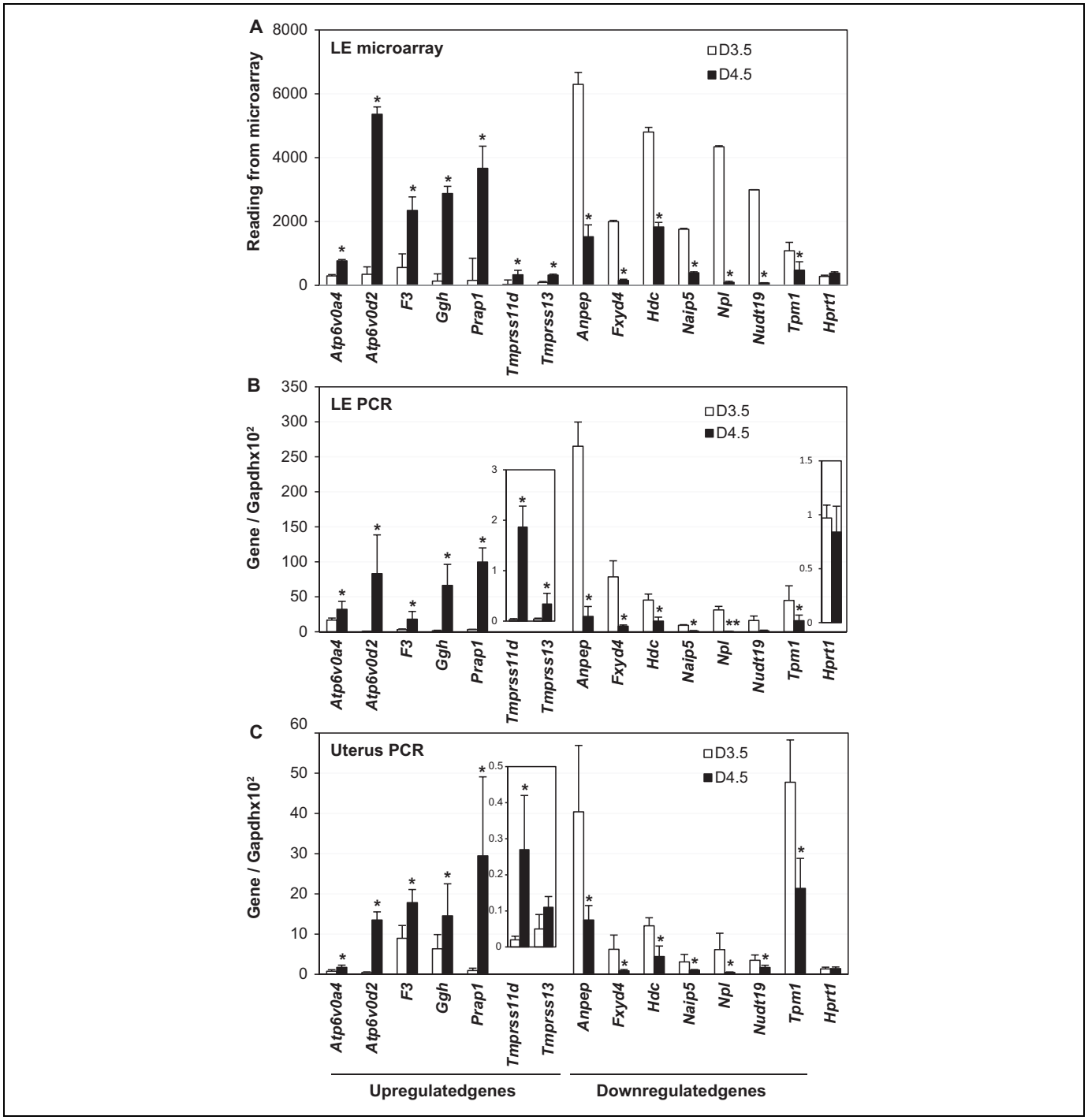

Figure 2. Expression of selected upregulated and downregulated genes. A, Readings from microarray analysis. N = 3. B, Real-time PCR of gestation day 3.5 (D3.5) and D4.5 LE. N = 5 to 6. C, Real-time PCR of D3.5 and D4.5uterus. N=5 to 6. A-C: *$P < .05$, compared to D3.5. Error bars represent standard deviation. *Atp6v0a4,* Atpase, H+ transporting, lysosomal V0 subunit A4; *Atp6v0d2*, Atpase, H+ transporting, lysosomal V0 subunit D2; *F3*, coagulation factor III; *Ggh*, γ-glutamyl hydrolase; *Prap1*, proline-rich acidic protein 1; *Tmprss11d*, transmembrane protease, serine 11d; *Tmprss13*, transmembrane protease, serine 13; *Anpep*, alanyl aminopeptidase; *Fxyd4*, FXYD domain-containing ion transport regulator 4; *Naip5*, nod-like receptor family, apoptosis inhibitory protein 5; *Npl*, N-acetylneuraminate pyruvate lyase; *Nudt19*, nucleoside diphosphate linked moiety X-type motif 19; *Tpm1*, tropomyosin 1; *Hprt1*, hypoxanthine phosphoribosyltransferase 1, a house keeping gene; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase, a house keeping gene as the loading control. PCR indicates polymerase chain reaction.

D4.5 LE. They were undetectable in other uterine compartments during periimplantation (Figure 3A-L). Although these 6 genes were all LE specific, there were differences in their expression patterns in the LE. *Atp6v0a4*, *Atp6v0d2*, and *F3* were expressed along the entire LE (Figure 3B, D, and F); *Tmprss11d* was mainly detected in the LE surrounding the embryo (Figure 3J),

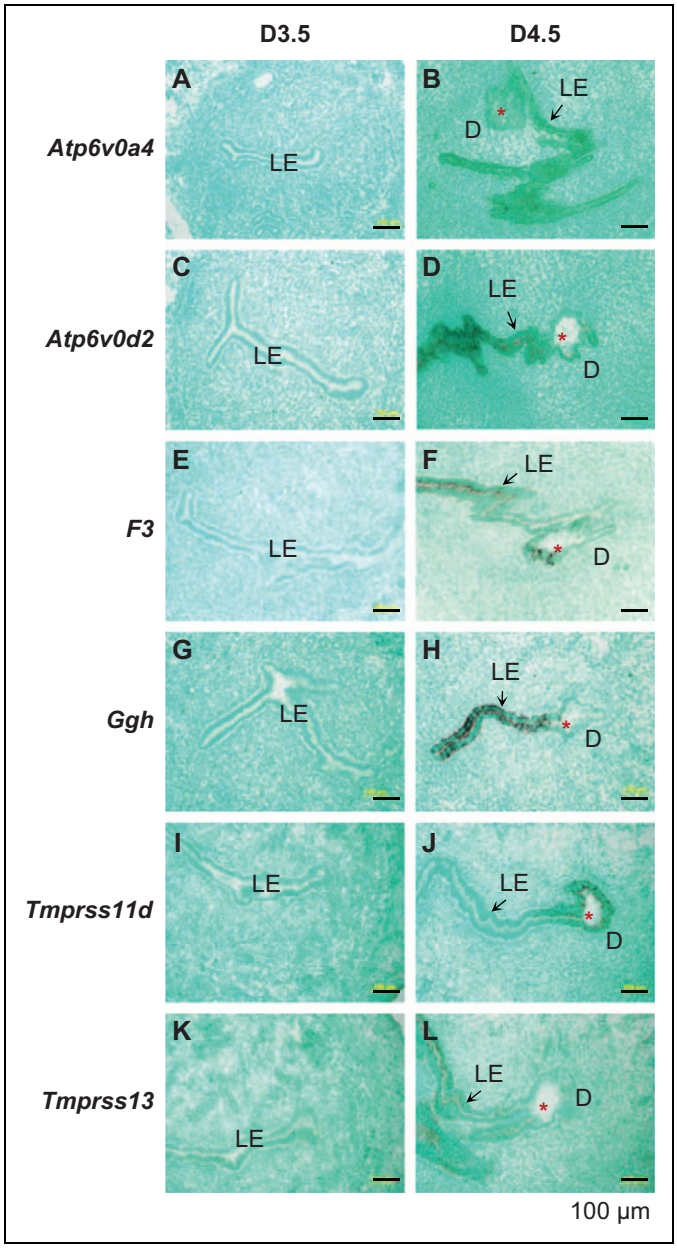

Figure 3. Localization of selected genes in the D3.5 and 4.5 mouse uterus by in situ hybridization using gene-specific antisense probes. These genes were shown upregulated in the D4.5 LE in microarray (Supplemental Table 2). A, *Atp6v0a4*, D3.5. B, *Atp6v0a4*, D4.5. C, *Atp6v0d2*, D3.5. D, *Atp6v0d2*, D4.5. E, *F3*, D3.5. F, *F3*, D4.5. G, *Ggh*, D3.5. H, *Ggh*, D4.5. I. *Tmprss11d*, D3.5. J, *Tmprss11d*, D4.5. K, *Tmprss13*, D3.5. L, *Tmprss13*, D4.5. D3.5, cross-sections (10 μm); D4.5, longitudinal sections (10 μm). No specific signal was detected using a sense probe (data not shown). Red star, embryo; LE, luminal epithelium; D, decidual zone; scale bar, 100 μm. N = 2 to 3.

and *Ggh* and *Tmprss13* had higher expression levels in the LE away from the implantation site (Figure 3H and L).

The expression levels of *Anpep*, *Fxyd4*, *Naip5*, *Npl*, *Nudt19*, and *Tpm1* were higher in the D3.5 LE compared to that in the D4.5 LE (Figures 4A-J and 5A and B), in which only *Nudt19* remained detectable in the D4.5 LE surrounding the embryo

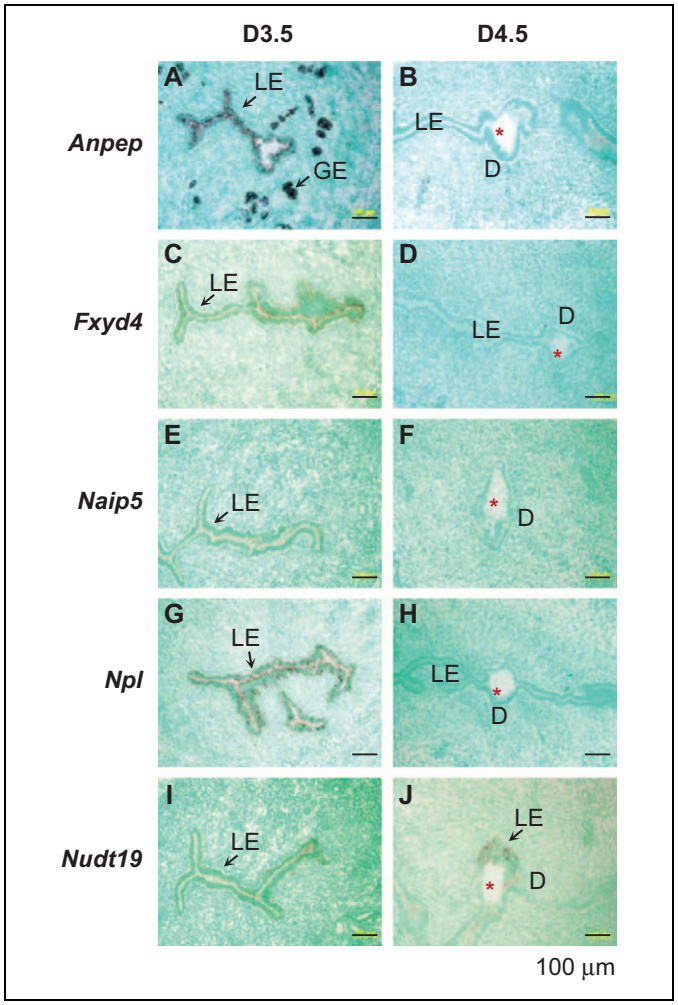

Figure 4. Localization of selected genes in the D3.5 and 4.5 uteri by in situ hybridization using gene-specific antisense probes. These genes were shown downregulated in the D4.5 LE in microarray (Supplemental Table 3). A, *Anpep*, D3.5. B, *Anpep*, D4.5. C, *Fxyd4*, D3.5. D, *Fxyd4*, D4.5. E, *Naip5*, D3.5. F, *Naip5*, D4.5. G, *Npl*, D3.5. H, *Npl*, D4.5. I, *Nudt19*, D3.5. J, *Nudt19*, D4.5. D3.5, cross-sections (10 μm); D4.5, longitudinal sections (10 μm). No specific signal was detected using a sense probe (data not shown). Red star, embryo; LE, luminal epithelium; GE, glandular epithelium; D, decidual zone; scale bar, 100 μm. N = 2 to 3.

(Figure 4J). Among these 6 genes, *Fxyd4*, *Naip5*, *Npl*, and *Nudt19* were LE specific in the periimplantation uterus. *Anpep* was also abundantly expressed in the D3.5 GE and disappeared from both LE and GE in the D4.5 uterus (Figure 4A and B). The spatiotemporal expression of *Tpm1* in the periimplantation uterus was unique, and it was highly expressed in the LE, GE, and myometrium of the D3.5 uterus (Figure 5A); upon embryo implantation, it disappeared from both LE and GE, remained in the myometrium, and appeared in the primary decidual zone of the D4.5 uterus (Figure 5B). Interestingly, among the 4 tropomysin isoforms TPM1-4,[60] *Tpm1* was the only one detected and differentially expressed in the periimplantation LE (Figure 5; Supplemental Table 3). *Tpm2* was detected only in the myometrium of the D3.5 uterus

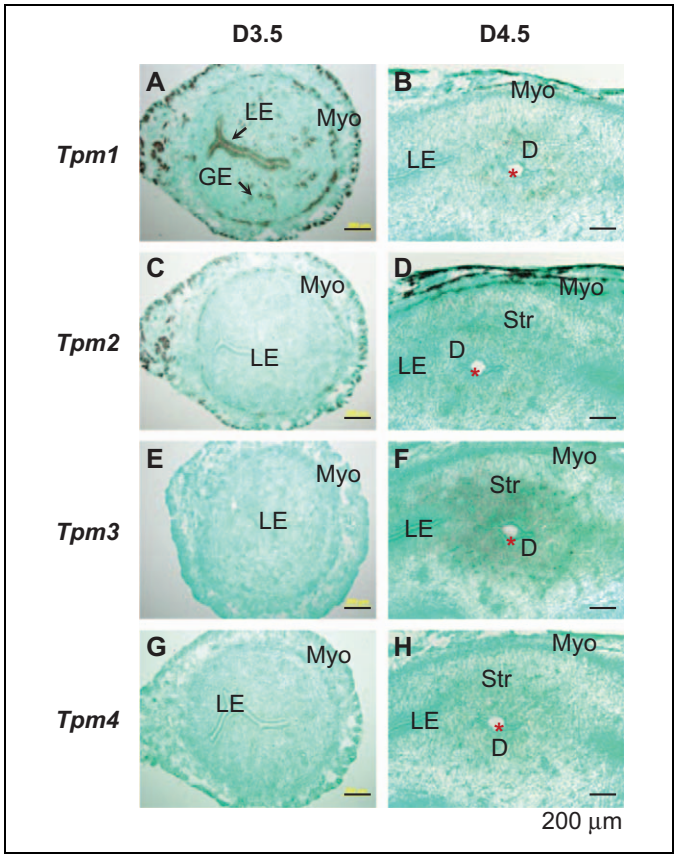

Figure 5. Localization of *Tpm1-4* in the D3.5 and D4.5 mouse uteri by in situ hybridization using *Tpm1*, *Tpm2*, *Tpm3*, and *Tpm4* antisense probes, respectively. A, *Tpm1*, D3.5. B, *Tpm1*, D4.5. C. *Tpm2*, D3.5. D. *Tpm2*, D4.5. E. *Tpm3*, D3.5. F. *Tpm3*, D4.5. G. *Tpm4*, D3.5. H, *Tpm4*, D4.5. D3.5, cross sections (10 μm); D4.5, longitudinal sections (10 μm). No specific signal was detected using a sense probe (data not shown). Red star, embryo; LE, luminal epithelium; GE, glandular epithelium; D, decidual zone; Myo, myometrium; scale bar, 200 μm. N = 2 to 3.

(Figure 5C) and remained in the myometrium and appeared in the stromal compartment at the implantation site of the D4.5 uterus (Figure 5D). *Tpm3* and *Tpm4* were undetectable in the D3.5 uterus (Figure 5E and G) but detectable in the stromal compartment at the implantation site of the D4.5 uterus (Figure 5F and H).

Discussion

General Discussion of the Approach

This microarray study identifies 627 differentially expressed genes in the periimplantation mouse LE, which include some well-known implantation markers in the LE but most of which have not been previously reported in the periimplantation LE. The inclusion of known implantation markers and the confirmation of previously uncharacterized genes in the LE demonstrate the effectiveness of the approach. Many stromal-specific genes upon implantation, such as *Bmp2*,[61] *Bmp7*,[61] *Fgf2*,[61] *Wnt4*,[34,61] *Wnt16*,[34] *Gja1*,[62] *Abp1*,[63,64] *Hand2*,[65] and so on, did not show differential expression in the periimplantation LE (GEO number: GSE44451), further supporting the efficiency of the LE microarray. However, 3 genes that were reported to be mainly detected in the stromal compartment upon implantation, *Gpx3*,[41] *Angpt2*,[66] and *Prss35*,[64] showed upregulation in the D4.5 LE (Supplemental Table 2). Differential expression of *Gpx3* and *Prss35* was confirmed by real-time PCR in D3.5 and D4.5 LE (data not shown). These observations suggest potential stromal cell contamination which would most likely occur during scraping of LE sheets with subepithelial stromal cells attached. Since many more stromal-specific genes do not show differential expression in the periimplantation LE, another explanation would be that there is true upregulation in the D4.5 LE, but the levels of upregulation are overshadowed by the much higher expression levels in the stromal compartment, and the probes used in the in situ hybridization were not sensitive enough to detect the differences in the LE but the stromal compartment.

Comparison With 3 Other Microarray Analyses Involved LE

There are three other microarray analyses involved LE.[27,28,37] These reported microarray analyses are very different from our study. Regardless, each one was compared with this study to obtain the overlapped genes (Table 2). Pan et al used ovariectomized nonpregnant mice treated with E2 and E2 + P4 to replicate the window of uterine receptivity. They only reported the genes upregulated in the E2 + P4-treated LE compared with those in E2-treated LE.[27] This mouse model may replicate the preimplantation uterine conditions from nonreceptive (E2-treated, ~D0) to receptive (E2 + P4-treated, ~D3.5 in this study), but it does not include the postimplantation D4.5 condition. There are 4 genes upregulated in both E2 + P4-treated LE and D4.5 LE, and 15 genes upregulated in E2+P4-treated LE but downregulated in D4.5 LE (Table 2). Niklaus et al compared the gene expression profiles between LE and GE from P4- and E2-treated ovariectomized early pregnant mice (delayed implantation model) before implantation initiation.[28] There are 28 genes shown in both LE versus GE array and D3.5 LE versus D4.5 LE array (Table 2). Chen et al compared the gene expression profiles in the postimplantation LE at implantation site and interimplantation site in the delayed implantation mouse model.[37] There are 20 genes overlapped with the results from our study. Since the study by Pan et al covers a different period from that in this study, and the studies by both Niklaus et al and Chen et al are spatial comparison but not temporal comparison as in this study (D3.5 LE vs D4.5 LE), the overlapped genes in Table 2 can only be instructive for periimplantation uterine gene expression studies.

About the Newly Characterized Genes

The 12 newly characterized genes have potential functions in ion transport (eg, *Atp6v0a4*, *Atp6v0d2*, *Fxyd4*), metabolism (eg, *Ggh*, *Npl*, *Nudt19*), morphology (eg, *Anpep*, *Tmprss11d*,

Tmprss13, Tpm1), immune responses (*Anpep*, *F3*), and apoptosis (eg, *Naip5*). The differential expression of these genes (Figures 2–5) and many others revealed in the microarray analysis (Supplemental Tables 2 and 3) indicates the involvement of the above-mentioned events in the LE, which are important for the establishment of uterine receptivity.

Membrane Transport and Metabolism in Periimplantation LE

Changes in membrane transport, including ion transport and metabolism in LE, could influence the uterine histotroph, a complex mixture of enzymes, growth factors, hormones, and nutrients that are critical for the activation of conceptus–endometrium interactions, embryo development, and implantation during early pregnancy.[67] Differential expression of transporters in ovine LE is involved in the altered nutrient profiles, such as glucose, amino acid, and ions in the uterine lumen during periimplantation.[68-71] Although the profiles of the components in the periimplantation mouse uterus lumen is unavailable, signaling pathway analysis of our microarray data indicates that two-third of the significantly changed pathways are related to metabolism (Table 1), and a large group of genes involved in transmembrane transport are differentially expressed in the periimplantation LE (Supplemental Tables 2 and 3). These molecular changes in the LE could affect the components in the uterine lumen to influence both the endometrium and the blastocyst for embryo implantation.

Morphological Changes in Periimplantation LE

Gene Ontology annotation indicates that several groups of differentially expressed genes could be involved in the LE morphological changes, such as proteolysis, regulation of cell adhesion, cytoskeleton organization, epidermal cell differentiation, and endocytosis. Cell adhesion is thought to play an important role in the initial attachment of the embryo to the LE.[72,73] Among the 15 genes in the category of regulation of cells adhesion (Supplemental Tables 2 and 3), secreted phosphoprotein 1, also called osteopontin, and its integrin receptors are among the best characterized cell adhesion molecules in the LE during embryo implantation.[72,73] Integrins are cell surface glycoproteins formed by noncovalent binding of α and β subunits. Interestingly, our LE microarray analysis reveals the differential expression of integrin α*1*, α*3*, and α*9* subunits in the periimplantation LE, suggesting that the integrin activity in the periimplantation LE might be regulated via α subunits (Supplemental Tables 2 and 3).

Immune Responses in Periimplantation LE

Immune responses play critical roles in embryo implantation.[74] Gene Ontology annotation groups 5 genes into ''immune response'' subcategory, *F3*, *Il1f6* (Interleukin 1 family, member 6), *Kng1* (Kininogen 1), *Mif* (macrophage migration inhibitory factor), and *Pla2g7* (phospholipase A2, group VII), which are all significantly upregulated in the D4.5 LE (Figure 3E and F and Supplemental Table 2). Besides the role of F3 in coagulation and proinflammation,[45] IL1F6 participates in cytokine/chemokine production[75]; KNG1 is also involved in coagulation and proinflammation[76]; MIF is a cytokine with chemokine-like functions mediating host immune and inflammatory response[77]; and PLA2G7 metabolizes platelet-activating factor and its roles in immune responses seem to depend on the specific biological settings.[78] The upregulation of these ''immune response'' genes indicates increased inflammatory responses in the LE during the early stages of embryo implantation.

Apoptosis in Periimplantation LE

Microarray analysis of human uterine epithelial cells between the late proliferative phase (pre-receptive) and the midsecretory phase (receptive for embryo implantation) indicates that cell cycle regulation is the most significantly enriched functional pathway in the late proliferative phase endometrial epithelium.[79] Cell cycle regulation does not appear in our Gene Ontology enrichment analysis (Supplemental Tables 2 and 3). It is reasonable because the time points selected in this study have passed the cell proliferation stage in the LE, which peaks at D1.5.[80] Instead, ''programmed cell death'' (apoptosis) is among the groups significantly downregulated in the D4.5 LE (Supplemental Table 3). Five genes in this group meet the selection criteria (fold change of ≥ 2, $P < .05$, and mean difference >200). The top 3 most differentially expressed genes in this group, *Naip1*, *Naip5* (Figure 4E and F), and *Naip7* encode apoptosis inhibitory proteins,[53] the fourth gene *Traf1* (TNF receptor-associated factor 1) is also a negative regulator of apoptosis.[81] The fifth gene in this group, *Xaf1* (XIAP associated factor 1), is an antagonist of XIAP anti-caspase activity.[82] It is expected that the net result of the downregulation of these 5 genes would be increased apoptosis, which is consistent with increased LE apoptosis in rodents during embryo implantation.[2,16] Caspase-3 was previously shown to be detectable in a few LE cells at the implantation site on D4.5 but undetectable in the LE of interimplantation site or D3.5 LE,[80] suggesting upregulation of caspase-3 in the D4.5 LE at the implantation site. Since the average readings of caspase-3 mRNA levels in the microarray are low (<200), it is possible that the main mechanism for LE apoptosis is regulation of caspase activity instead of mRNA levels, for example, through downregulation of apoptosis-negative regulators in the LE.

Potential Mechanisms of Gene Regulation in Periimplantation LE

Gene Ontology annotation reveals that the largest group of both upregulated and downregulated genes is involved in regulation of transcription (Figure 1; Supplemental Tables 2 and 3), indicating that transcriptional regulation is most likely the main mechanism for the molecular changes in the LE during periimplantation. Interestingly, ''miRNA regulation of DNA damage response'' is among the significantly changed signaling

pathways (Table 1), and 9 genes in "chromosome organization" category are downregulated in the D4.5 LE (Supplemental Table 3), suggesting that epigenetic mechanisms, such as miRNAs and chromosome organization, could also be involved in regulating the molecular changes in the periimplantation LE. This speculation is supported by the observation that several miRNAs and their predicated target genes are differentially expressed in the human uterine epithelium during estrous cycle.[79] In addition to continuous research on understanding the LE gene network in the establishment of uterine receptivity and how LE communicates with other uterine compartments in the establishment of uterine receptivity, research on understanding the molecular mechanisms, including epigenetic mechanisms, of how the LE gene network is regulated during initial implantation stages is another important direction for deciphering uterine function in embryo implantation.

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Authors' Note

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Supplemental Material

The online tables are available at http://XXX.sagepub.com/supplemental.

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